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Purification and Characterization of α-Amylase from *Bacillus licheniformis* CUMC305

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α-Amylase produced by *Bacillus licheniformis* CUMC305 was purified 212-fold with a 42% yield through a series of four steps. The purified enzyme was homogeneous as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and discontinuous gel electrophoresis. The purified enzyme showed maximal activity at 90°C and pH 9.0, and 91% of this activity remained at 100°C. The enzyme retained 91, 79, and 71% maximal activity after 3 h of treatment at 60°C, 3 h at 70°C, and 90 min at 80°C, respectively, in the absence of substrate. On the contrary, in the presence of substrate (soluble starch), the α-amylase enzyme was fully stable after a 4-h incubation at 100°C. The enzyme showed 100% stability in the pH range 7 to 9; 95% stability at pH 10; and 84, 74, 68, and 50% stability at pH values of 6, 5, 4, and 3, respectively, after 18 h of treatment. The activation energy for this enzyme was calculated as 5.1×10^5 J/mol. The molecular weight was estimated to be 28,000 by sodium dodecyl sulfate-gel electrophoresis. The relative rates of hydrolysis of soluble starch, amylose, amylopectin, and glycogen were 1.27, 1.8, 1.94, and 2.28 mg/ml, respectively. V_{max} values for hydrolysis of these substrates were calculated as 0.738, 1.08, 0.8, and 0.5 mg of maltose/ml per min, respectively. Of the cations, Na^+, Ca^{2+}, and Mg^{2+}, showed stimulatory effect, whereas Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Fe^{2+}, Co^{2+}, Cd^{2+}, Al^{3+}, and Mn^{2+} were inhibitory. Of the anions, azide, F^-, SO_3^{2-}, SO_4^{3-}, $S_2O_3^{2-}$, MoO_4^{2-}, and Wo_4^{2-} showed an excitant effect. *p*-Chloromercuribenzoic acid and sodium iodoacetate were inhibitory, whereas cysteine, reduced glutathione, thiourea, β-mercaptoethanol, and sodium glycerophosphate afforded protection to enzyme activity. α-Amylase was fairly resistant to EDTA treatment at 30°C, but heating at 90°C in presence of EDTA resulted in the complete loss of enzyme activity, which could be recovered partially by the addition of Cu^{2+} and Fe^{2+} but not by the addition of Ca^{2+} or any other divalent ions.

The occurrence of microbial amylases with varied catalytic properties has recently been extensively reviewed (11). The majority of thermostable α-amylases from *Bacillus* spp. heretofore purified, have shown maximal activity in the acidic to neutral pH range (2, 4, 8, 20, 21, 31, 32; A. M. Joyce, Ph.D. thesis, National University of Ireland, Dublin, 1977). Purified α-amylases of *Bacillus* spp. which are active under alkaline conditions have been found to be unstable at temperatures above 50°C (3, 13) or 60°C (19). A thermostable alkaline α-amylase from *Bacillus licheniformis* has been purified (23) which has maximal activity at 76°C. α-Amylase from *B. licheniformis* CUMC305, a new strain reported earlier from this laboratory (18), was purified to homogeneity, and the enzyme showed maximal activity at 90°C and pH 9, as reported for *B. licheniformis* NCIB6346 (19). However, 91% of the maximal α-amylase activity was observed even at 100°C in the case of *B. licheniformis* CUMC305, unlike the sharp fall of enzyme activity reported for *B. licheniformis* NCIB6346 (19). In the absence of substrate, this enzyme was highly stable even at 80°C and pH 10, whereas the purified α-amylases of *B. licheniformis* 584 (23) or *B. licheniformis* NCIB6346 (19) were very unstable at temperatures beyond 60°C and at pH values below 7 or above 9. The α-amylase of *B. licheniformis* CUMC305 was remarkably stable at 100°C in the presence of soluble starch. The enzyme reported here showed several other interesting characteristics previously unreported from other thermophilic strains of *B. licheniformis*. Because of the immense potential of this enzyme for industrial applications, a preliminary study had been undertaken (5). The current report deals with the purification and catalytic and molecular properties of the thermostable *B. licheniformis* CUMC305 α-amylase, including the effects of cations and anions on enzyme activity. This

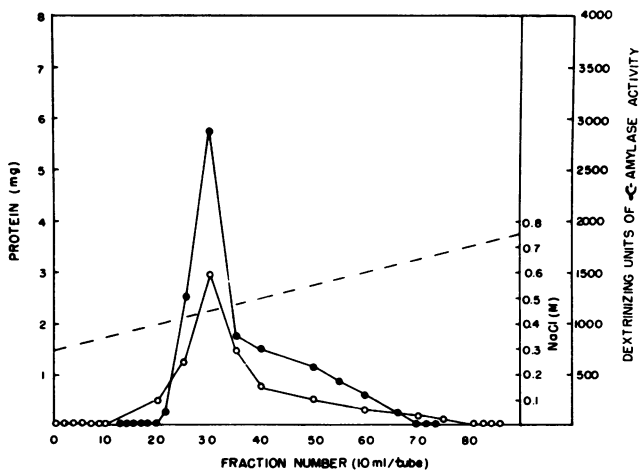

FIG. 1. Elution pattern of carboxymethylcellulose column chromatograph of the *B. licheniformis* CUMC305 α-amylase. Symbols: ●, dextrinizing activity; ○, protein; ---, NaCl.

study was planned to enable the adoption of this enzyme in suitable industries, in which thermostable α-amylases are used in large quantities (10, 24).

MATERIALS AND METHODS

Organism. The *B. licheniformis* CUMC305 isolate described previously (18) was used in this study.

Medium composition and culture conditions. The growth medium used for α-amylase production was composed of: 0.2% soluble starch, 0.2% $(NH_4)_2HPO_4$, 0.05% Na_2CO_3, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $MnCl_2 \cdot 4H_2O$, 0.02% K_2SO_4, 0.4% cornsteep liquor, 0.2% beef extract, and 0.5% peptone in distilled water. This medium was supplemented with 0.5% defatted groundnut meal for improved α-amylase production (14). The medium was adjusted to pH 6.7 and autoclaved at 15 lbs/in^2 pressure for 20 min. Roux bottles (1,000 ml) containing 150 ml of medium were inoculated with a 14-h washed culture (1.68×10^6 cells). Cultures were incubated at 48°C for 24 h under stationary-phase conditions for optimum enzyme production.

Assay of α-amylase. The dextrinizing activity was measured by the method of Smith and Roe (26). One dextrinizing unit was equivalent to the amount of enzyme which hydrolyzed 1 mg of soluble starch in 5 min. Saccharolytic activity was measured as an increase in reducing power by using the method of Sumner, as modified by Bernfeld (1); 1 U of α-amylase activity was equivalent to the amount of enzyme which released 1 mg of reducing sugar as maltose in 5 min. The reaction mixture for a standard assay was 0.5 ml of 1% soluble starch, 0.4 ml of buffer (0.05 M glycine-sodium hydroxide) and 0.1 ml of enzyme. In the control assay, enzyme was replaced by 0.1 ml of distilled water. After incubation for 5 min at 90°C, the reaction was stopped with 0.5 ml of 1 N HCl to determine the dextrinizing activity. Next, 0.1 ml of the reaction mixture was added to 14.3 ml of distilled water, 0.5 ml of 1 N HCl, and 0.1 ml of 0.3% I_2–3% KI solution, and readings were then taken in a Klett-Summerson colorimeter fitted with red filter (no. 66). For measurement of the saccharolytic activity, the reaction mixture was as described above, but in the control assay, the enzyme (0.1 ml) was added after addition of 1 ml of 1% dinitrosalicylic acid reagent prepared as described previously (14) and normally used to stop the reaction. A blank was prepared with 1 ml of distilled water and 1 ml of 1% dinitrosalicylic acid reagent. The contents of all assay tubes were diluted with 10 ml of distilled water, and readings were taken in the Klett-Summerson colorimeter fitted with a green filter (no. 54).

Amylase purification. The culture broth was centrifuged at 10,000 rpm for 15 min at room temperature

TABLE 1. Purification and overall recovery of *B. licheniformis* CUMC305 α-amylase

Purification step	Vol (ml)	Total dextrinizing α-amylase units	Total protein (mg)	Sp act[a]	% Yield	Purification (fold)
Original broth	950	17,378	4,168	4.17	100	1
Condensed broth	290	13,330	2,666	5	76.7	1.2
30 to 65% ammonium sulfate precipitation	20	8,960	320	28	51.6	6.7
Dialysis	20	8,750	250	35	50.35	8.4
Carboxymethylcellulose column chromatography	700	7,300	8.27	882.7	42.0	211.67

[a] Dextrinizing units of α-amylase per milligram of protein.

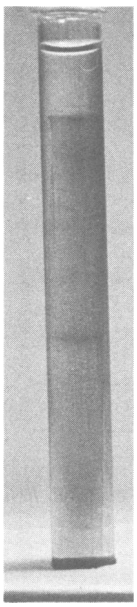

FIG. 2. Polyacrylamide gel electrophoresis of α-amylase of *B. licheniformis* CUMC305.

(30°C), and the clear supernatant was pooled together. The supernatant was then condensed in a rotary evaporator under vacuum at 10°C to reduce the total volume and to concentrate the enzyme protein. The condensed broth was then placed for 30 min in a water bath maintained at 80°C to precipitate out some undesired proteins. The precipitate was discarded after centrifugation. The supernatant with enzyme was chilled, and solid ammonium sulfate was added with gentle stirring to 30% saturation; after centrifugation, the precipitate was discarded. More ammonium sulfate was added to the supernatant to 65% saturation. The precipitate was collected and dissolved in 0.01 M sodium phosphate buffer (pH 6.4) and dialyzed overnight against the same buffer. After dialysis, the enzyme solution was applied to a column of carboxymethylcellulose (53 by 3 cm; bed volume, 50 ml) which had been equilibrated with 400 ml of 0.01 M sodium phosphate buffer (pH 6.4). The column was next rinsed with 200 ml of the same buffer (pH 6.4) to wash out the unadsorbed material. Subsequently, the enzyme was eluted with 200-ml volumes of the same buffer containing a linear gradient of NaCl (between 0.1 and 0.8 M NaCl) at a flow rate of 40 ml/h. Fractions (10 ml) were collected, and the enzyme was eluted between 0.3 and 0.5 M NaCl. Active fractions were pooled (700 ml), condensed to about 120 ml, and dialyzed overnight against distilled water.

Estimation of protein. Protein was estimated according to the method of Lowry et al. (16), with crystalline bovine serum albumin (Sigma Chemical Co.) as the standard.

Determination of molecular weight. The sodium dodecyl sulfate-polyacrylamide gel method of Weber and Osborn (30) was used. Gels (7.5%) were prepared in tubes (8 by 0.5 cm). Protein samples were prepared by method 1 in the presence of sodium dodecyl sulfate at 100°C for 3 min. Electrophoresis was performed with the positive electrode in the lower chamber. A 1-mA current was applied per tube, until the tracking dye entered the sample gel (1 h); the current was then raised to 3 mA per tube for the remainder of the run. Gels were stained with Coomassie brilliant blue R250 (Bio-Rad Laboratories) and destained by diffusion. Proteins of approximately known molecular weights (bovine serum albumin [66,000], egg albumin [45,000], pepsin [34,700], trypsinogen [phenylmethylsulfonyl fluoride-treated; 24,000], β-lactoglobulin [18,400], and lysozyme [14,300]) from Sigma Chemical Co. served as reference markers.

Disc electrophoresis. Polyacrylamide gels prepared by the method of Davis (7) were used in discontinuous gel electrophoresis apparatus. Standard 7% gels were prepared in tubes (8 by 0.5 cm) and run at pH 8.3 in Tris-glycine buffer. Samples (0.05 ml) containing about 200 μg of protein were mixed with the sample gel solution and applied onto the stacking gel. A 1-mA current was applied to each tube until the tracking dye entered the separating gel; the current was then raised to 3 mA per tube for the remainder of the run (ca. 1.5 h). Gels were stained with amido black (0.5%) for 4 h and destained by diffusion with 7% acetic acid. Duplicate unstained gels were sliced into sections corresponding to the electrophoretic pattern of stained gels, macerated in tubes containing 2 ml of 0.05 M glycine-sodium hydroxide buffer (pH 9), stored overnight at 10°C, and assayed for α-amylase activity. Entire unstained gel columns were separately placed for 3 min on 1% soluble starch-agar petri dishes which were

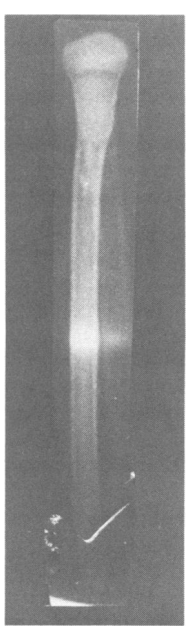

FIG. 3. Starch hydrolysis pattern of polyacrylamide gel with purified enzyme. The white zone represents area of enzymatically hydrolyzed starch against the dark background of the starch-iodide complex.

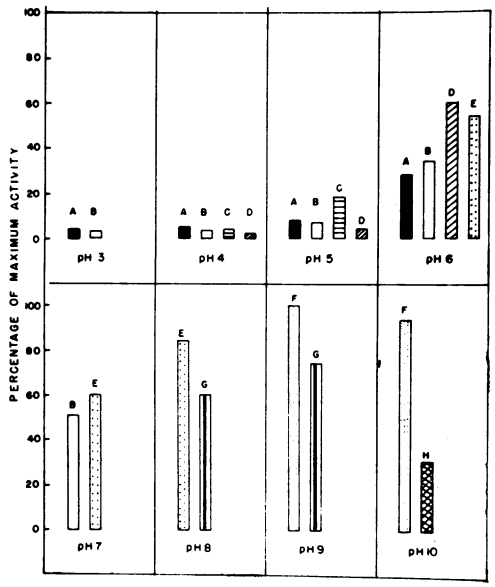

FIG. 4. Effect of different buffers and pH values on *B. licheniformis* CUMC305 α-amylase dextrinizing activity. Reaction mixtures containing 0.5 ml of soluble starch, 1% (wt/vol) of a 0.1-ml enzyme solution, and 0.4 ml of one of the buffers each (A to H) were assayed at 90°C for 5 min. The relative activity was calculated as a percentage of the maximal dextrinizing activity observed with 0.05 M glycine-sodium hydroxide buffer (pH 9.0) taken as 100%. Buffers: A, sodium citrate-citric acid (0.05 M); B, citric acid-disodium phosphate (0.15 M); C, acetic acid-sodium acetate (0.1 M); D, succinic acid-sodium hydroxide (0.1 M); E, sodium dihydrogen phosphate-disodium hydrogen phosphate (0.1 M); F, glycine-sodium hydroxide (0.05 M); G, Tris-hydrochloride (0.1 M); and H, sodium carbonate-sodium bicarbonate (0.1 M).

being incubated at 90°C in a hot-air oven for 5 min. Subsequently, after the gels were removed, each petri dish was cooled and flooded with iodine solution (0.3% $I_2$3% KI). A transparent band was visible against the dark-blue background of the starch-iodide complex, indicating that the starch had been hydrolyzed by the enzyme in that portion of the gel.

RESULTS

Purification of α-amylase. Heating of the condensed enzyme extract at 80°C for 30 min denatured and precipitated heat-labile interfering proteins; the total dextrinizing activity of α-amylase in the supernatant remained unchanged. Subsequent fractionation with ammonium sulfate resulted in a high yield (51.6% of original activity) of α-amylase with increased specific activity. The majority of enzyme activity was found in the 30 to 65% fraction. After dialysis, this fraction was chromatographed on a carboxymethylcellulose column (Fig. 1). The enzyme was eluted at between 0.3 and 0.5 M NaCl in 70 10-ml fractions. The results of the overall purification procedure are presented in Table 1. The α-amylase of *B. licheniformis* CUMC305 was purified ca. 212-fold, with an overall yield of 42%. The active fractions were pooled together and concentrated for further studies. Discontinuous electrophoresis of the sample of concentrate from the carboxymethylcellulose column, on 7% polyacrylamide gels, showed a single protein band after staining with 0.5% amido black (Fig. 2). An unstained gel, incubated on a 1% soluble starch-agar petri dish, also showed a single band of hydrolyzed starch corresponding to the band pattern on the stained gel (Fig. 3).

Properties of the enzyme. (i) Effect of temperature on enzyme activity and stability. The effect of temperature on α-amylase, with respect to enzyme dextrinizing activity at pH 9, showed 90°C as the optimum temperature. The enzyme showed 50% of the maximum activity at 55°C and 91% at 100°C. The effects of temperature on initial substrate hydrolysis velocities were studied over the range of 10 to 90°C in 10-degree increments. By a conventional Arrhenius plot of the date, the activation energy was calculated to be ca. 5.1×10^5 J/mol. α-Amylase was 100% stable for 3 h at temperatures below 50°C. At 60 and 70°C stability declined after 1 h to 91% and 79%, respectively, after 3 h. At 80°C, the enzyme was fully stable for 45 min; at 90°C, the dextrinizing activity was rapidly lost in the absence of substrate. However, when soluble starch was provided, *B. licheniformis* CUMC305 α-amylase was not inactivated even after a 4-h incubation at 100°C. The relative activity after a 1-h incubation was recorded as 136% of the normal activity (assayed after 5 min) and ca. 191% after a 4-h incubation at 100°C and pH 9.

(ii) Effect of pH on enzyme activity and stability. The effect of different buffers and pH on dextrinizing activity of α-amylase is shown in Fig. 4. The enzyme was active over the entire range from pH 3 to pH 10, with maximal activity at pH 9, by using 0.05 M glycine-sodium hydroxide buffer. Variation of the buffers exerted marked effects on enzyme activity at different pH values. Tris-hydrochloride and carbonate-bicarbonate buffers lowered enzyme activity.

Enzyme activity was found to be stable in the neutral to alkaline pH range (pH 7 to 9) after an 18-h treatment at 30°C. It was also interesting that 50% of the α-amylase activity was retained at pH 3, 66% was retained at pH 4, 75% was retained at pH 5, and 85% was retained at pH 6 after 18 h. A sharp decline in activity at pH values of 3, 4, and 5 was noticed within 15 min, but then the activity remained the same for at least 2 h. Further deterioration occurred when samples were incubated overnight.

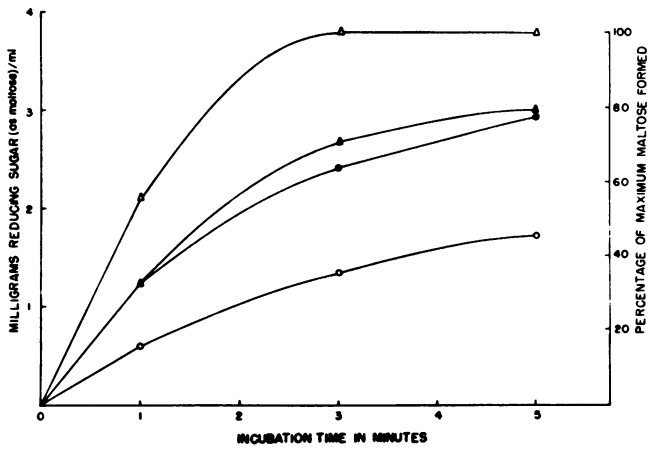

FIG. 5. Time course of reducing-sugar liberation from different substrates (1% [wt/vol]) by α-amylase of *B. licheniformis* CUMC305, under routine assay conditions of 90°C and pH 9.0. Symbols: ●, soluble starch; △, amylose; ▲, amylopectin; and ○, glycogen.

Estimation of molecular weight. The electrophoretic mobility profiles of known-molecular-weight proteins were compared with that of the purified α-amylase of *B. licheniformis* CUMC305 in sodium dodecyl sulfate-gel electrophoresis, and the molecular weight of α-amylase was calculated to be 28,000 at pH 7.

Substrate specificity. The substrate specificity of purified α-amylase from *B. licheniformis* CUMC305 is shown in Fig. 5. From 1% concentrations of the different substrates, release of reducing sugar (as maltose) was very rapid from amylose (129%) but was slower from soluble starch (100%), amylopectin (101.4%), and glycogen (58.3%).

The values for K_m and V_{max} were determined from the Lineweaver-Burk plot; V_{max} values for hydrolysis of soluble starch, amylose, amylopectin, and glycogen were calculated as 0.738, 1.08, 0.8, and 0.5 mg maltose per ml per min, respectively. K_m values for soluble starch, amylose, amylopectin, and glycogen were calculated as 1.274, 1.818, 1.94, and 2.28 mg/ml, respectively.

Effects of cations, anions, and other reagents on α-amylase activity. The dextrinizing activity was accelerated in the presence of selected cations and nonmetallic reagents (Table 2). Sulphydryl reacting reagent, e.g., *p*-chloromercuribenzoic acid was strongly inhibitory, whereas glutathione (reduced), thiourea, and β-mercaptoethanol protected enzyme activity. The capability of cysteine to promote activity and partially reverse the inhibition by $HgCl_2$ was also observed. The metal ions-EDTA treatment at low temperatures (≤30°C) effected small losses of enzyme activity (Table 2). However, it was seen that at 90°C, the enzyme was completely inhibited by EDTA treatment. The metal ions Cu^{2+} and Fe^{2+} could partially revive enzyme activity, but no other cation showed any stimulatory effect. The effect of anions on the dextrinizing activity of α-amylase is shown in Table 3. The enzyme also retained activity in the presence of iodoacetate at a low concentration of 10 mM but was inhibited by higher concentrations around 100 mM. However, PO_4^{3-} was stimulatory at high concentrations (ca. 100 mM), whereas molybdate was beneficiary from low to high concentrations, i.e., between 10 and 100 mM. There was no change in α-amylase activity with the presence of either citrate or arsenite anions.

DISCUSSION

Upon purification, The α-amylase of *B. licheniformis* CUMC305 showed a single protein band, in contrast to the four active protein bands of α-amylase purified from *B. licheniformis* 584 (23).

The alkaline α-amylases of *Bacillus* spp. (3, 13) were not thermostable, whereas α-amylases from *B. licheniformis* 584 and *B. licheniformis* NCIB6346 (19) showed optimal activity at 76 and 92°C, respectively, retaining substantial activity in the alkaline pH range, but were unstable beyond 60°C. On the other hand, highly thermostable β-amylases were found to be inactive under alkaline conditions (17, 20, 21, 31). *B. licheniformis* CUMC305 α-amylase proved to be highly thermostable, showing optimal dextrinizing activity at 90°C and 91% of optimal activity at 100°C, which clearly showed that the enzyme was different from the enzyme reported from *B. licheniformis* NCIB6346 (19) *B. licheniformis* CUMC305 α-amylase has a very broad pH activ-

TABLE 2. Effect of cations and nonmetallic reagents on dextrinizing activity of *B. licheniformis* CUMC305 α-amylase[a]

Treatment	Residual activity (%) at indicated test reagent concn (mM)		
	0	0.5	4
NaCl	100	121	119
Na^+ + EDTA	—	59	—
$CaCl_2$	100	119	117
Ca^{2+} + EDTA	—	57	—
$MgCl_2 \cdot 6H_2O$	100	114	112
Mg^{2+} + EDTA	—	72	—
$AlCl_3$	100	117	—
Al^{3+} + EDTA	—	78	—
$MnCl_2 \cdot 4H_2O$	100	102	37
Mn^{2+} + EDTA	—	63	—
$3CdSO_4 \cdot 8H_2O$	100	80	4
Cd^{2+} + EDTA	—	117	—
$CoCl_2$	100	102	13
Co^{2+} + EDTA	—	76	—
$FeCl_3$	100	93	10
Fe^{3+} + EDTA	—	63	—
AgCl	100	47	—
Ag^+ + EDTA	—	53	—
$ZnSO_4 \cdot 7H_2O$	100	84	—
Zn^{2+} + EDTA	—	59	—
$NiSO_4 \cdot 6H_2O$	100	63	—
Ni^{2+} + EDTA	—	47	—
$CuSO_4 \cdot 5H_2O$	100	58	—
Cu^{2+} + EDTA	—	55	—
$HgCl_2$	100	—	—
Hg^{2+} + EDTA	—	63	—
p-Chloromercuribenzoic acid	100	—	—

[a] Reaction mixtures contained 0.1 ml of enzyme solution, 0.1 ml of test reagent, 0.3 ml of 0.05 M glycine-sodium hydroxide (pH 9.0), and 0.5 ml of soluble starch (1% [wt/vol]); the assay was conducted at pH 9.0 and 90°C. For study of the effects of the metal ion-EDTA combination, the enzyme was first incubated for 10 min in the presence of 0.5 mM metallic reagent alone and subsequently treated with 1 mM EDTA for 10 min at room temperature (30°C). The residual activity was assayed as described in the text and is expressed as a percentage of the activity of the untreated control (taken as 100%). One dextrinizing unit was equivalent to the amount of enzyme which hydrolyzed 1 mg of soluble starch in 5 min.

ity curve (pH 3 to 10). In addition, the enzyme was highly stable up to 80°C, beyond which, even at 100°C, the substrate (soluble starch) afforded total protection for at least 4 h. The enzyme was also highly stable under acidic, neutral, or alkaline pH conditions and was therefore strikingly different from the enzymes from all other previously reported strains of *B. licheniformis* (6, 17, 23, 25).

The molecular weight of the α-amylase (28,000) from *B. licheniformis* CUMC305 was higher than that reported for *B. licheniformis* 584 α-amylase (22, 500) (23) or *B. licheniformis* BLMI777 α-amylase (23,500) (6). Therefore, it is evident that the molecular weight of the α-amylase from *B. licheniformis* strains was much lower than the average molecular weight (50,000) of other bacterial α-amylases (15) and closely resembled the molecular weight (24,000) of the basic subunit of *Bacillus subtilis* α-amylase (22). The relative rates of substrate hydrolysis indicated that amylose was more easily hydrolyzed than was amylopectin or glycogen, because α-1,4 linkages were attacked more easily than were α-1,6 linkages. The dextrinizing power of the enzyme was stimulated by the presence of the cations Na^+, Ca^{2+}, Mg^{2+}, whereas Zn^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Al^{3+}, Mn^{3+}, and the heavy metals Ag^+, Cu^{2+}, and Hg^{2+} were inhibitory, as has been reported for other α-amylases (12, 29). The enzyme retained full activity after continuous dialysis against distilled water, and the addition of calcium or other divalent cations had no remarkable effect on enzyme activity or stability, unlike traits reported for other α-amylase enzymes (27). On the contrary, excess calcium (10 mM) was unfavorable for α-amylase activity. α-Amylase activity was inhibited by the sulphydryl reacting reagent *p*-chloromercuribenzoic acid, whereas

TABLE 3. Effect of inorganic and organic anions on the dextrinizing activity of *B. licheniformis* CUMC305 α-amylase[a]

Treatment	Activity (%) at indicated test reagent concn (mM)[b]	
	10	100
NaOH	110	95
$NaHCO_3$	102	71
NaCl	102	93
$NaNO_2$	110	83
$NaNO_3$	105	88
NaN_3	110	110
NaF	105	110
Na_2CO_3	102	83
Na_2SO_3	115	115
Na_2SO_4	117	110
$Na_2S_2O_3 \cdot 5H_2O$	110	117
Potassium ferricyanide	105	57
Sodium iodoacetate	103	20
Sodium glycerophosphate	107	115
Sodium tungstate	121	121

[a] A mixture of 0.1 ml of enzyme solution, 0.1 ml of reagent, 0.3 ml of 0.05 M glycine-sodium hydroxide (pH 9.0), and 0.5 ml of soluble starch (1% [wt/vol]) was assayed for 5 min at 90°C. The activity is expressed as a percentage of the activity of the untreated control, which is assumed as 100%.

[b] At a test reagent concentration of 0 mM (control), the α-amylase activity was 100% for all treatments.

thiol compounds afforded protection. This suggests that the -SH group of the enzyme protein is responsible for normal catalytic activities. Enzyme activity was unaffected by low concentrations of EDTA (1 mM) at 30°C, as reported for *B. licheniformis* 584 (23), *Bacillus* spp. strain NRRL-B (3), and *B. subtilis* var. *amylosaccharicticus* (28); however, the presence of EDTA at 90°C was detrimental to α-amylase activity. The resultant denaturation due to the combined effect of EDTA and heat was comparable to earlier observations with α-amylase from other sources (9, 21). However, for *B. licheniformis* CUMC305 α-amylase, the cations Cu^{2+} and Fe^{2+}, if added subsequently, could partially revive enzyme activity. It was interesting that *B. licheniformis* CUMC305 α-amylase was differentially influenced by the addition of various monovalent, divalent, or trivalent anions. The dextrinizing activity of the enzyme was enhanced by the anions in this order: SO_4^{2-} > OH^- > NO_2^- > F^- > NO_3^- > ferricyanide > molybdate > acetate > Cl^- > CO_3^{2-} > HCO_3^- > iodoacetate at 10 mM concentrations. High concentrations (100 mM) were injurious in certain cases. The trivalent anion PO_4^{3-} was beneficiary at 100 mM, whereas azide, SO_3^{2-}, $S_2O_3^{2-}$, glycerophosphate, and tungstate were equally suitable at 10 or 100 mM concentrations. Arsenite and citrate had no effect upon α-amylase activity of *B. licheniformis* CUMC305. Therefore, the thermostable α-amylase released by *B. licheniformis* CUMC305 studied from several perspectives and reported here is characteristically different from the α-amylase enzyme of the other *B. licheniformis* strains or microbial α-amylases reported earlier.

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